

probes. A double stranded probe may then be obtained by either synthesizing the complementary strand and hybridizing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence. Such cDNA probes may be used in the design of oligonucleotide probes and primers for screening and cloning such genes, e.g., using well known PCR techniques, or, alternatively, may be used to detect the presence or absence of a PfEMP1 gene in a cell. Such nucleic acids, or fragments may comprise part or all of the cDNA sequence that encodes the polypeptides of the present invention. Effective cDNA probes may comprise as few as 15 consecutive nucleotides in the cDNA sequence, but will often comprise longer segments. Further, these probes may further comprise an additional nucleotide sequence, such as a transcriptional primer sequence for cloning, or a detectable group for easy identification and location of complementary sequences.

cDNA or genomic libraries of various types may be screened for new alleles or related sequences using the above probes. The choice of cDNA libraries normally corresponds to tissue sources which are abundant in mRNA for the desired polypeptides. Phage libraries are normally preferred, e.g., λ gt11, but plasmid or YAC libraries may also be used. Clones of a library are spread onto plates, transferred to a substrate for screening, denatured, and probed for the presence of the desired sequences.

In a related aspect, the nucleic acids of the present invention also include the PCR product or RT-PCR product, produced using the above described primer probes. For example, primer probes derived from the nucleotide sequence shown, described &/or referenced herein (including incorporated by reference), may be used to amplify sequences from different malaria parasites, and in particular, different strains of *P. falciparum*.

The nucleic acids of the present invention may be present in whole cells, cell lysates or in partially pure or substantially pure or isolated form. Such "substantially pure" or "isolated" forms of these nucleic acids generally refer to the nucleic acid separated from contaminants with which it is generally associated, e.g., lipids, proteins and other nucleic acids. The nucleic acids of the present invention will be greater than about 50% pure. Typically, the nucleic acids will be more than about 60% pure, more typically, from about 75% to about 90% pure, and preferably, from about 95% to about 98% pure.

The present invention also provides substantially similar nucleic acid sequences, allelic variations and natural or induced sequences of the above described nucleic acids, as

well as chemically modified and substituted nucleic acids, e.g., those which incorporate modified nucleotide bases or which incorporate a labeling group. In addition to comprising a segment which encodes a PfEMP1 protein or fragment thereof, the nucleic acids of the present invention may also comprise a segment encoding a heterologous protein, such that the gene is expressed to produce the two proteins as a fusion protein, as substantially described above.

In addition to their use as probes, the nucleic acids of the present invention may also be used in the preparation of the polypeptides of the present invention, as described above. DNA encoding the polypeptides of the present invention will typically be incorporated into DNA constructs capable of introduction to and expression in an *in vitro* cell culture. Often, the nucleic acids of the present invention may be used to produce a suitable recombinant host cell.

Specifically, DNA constructs will be suitable for replication in a unicellular host, such as bacteria, e.g., *E. coli*, viruses or yeast, but may also be intended for introduction into a cultured mammalian, plant, insect, or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA segment encoding the desired polypeptide, transcriptional and translational initiation and termination regulatory sequences operably linked to the polypeptide encoding segment. A DNA segment is operably linked when it is placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence; DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof. The selection of an appropriate promoter sequence will generally depend upon the host cell selected for the expression of the DNA segment.

Examples of suitable promoter sequences include prokaryotic, and eukaryotic promoters well known in the art. See, e.g., Sambrook et al., *supra*. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is

recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known and available. See Sambrook et al., supra.

Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the PfEMP1 polypeptide encoding segment may be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al., supra, and in Metzger et al., Nature 334:31-36 (1988).

The vectors containing the DNA segments of interest, e.g., those encoding polypeptides comprising a PfEMP1 protein or fragments thereof, can be transferred into the host cell by well known methods, which may vary depending upon the type of host used. For example, calcium chloride transfection is commonly used for prokaryotic cells, whereas calcium phosphate treatment may be used for other hosts. See, Sambrook et al., supra. The term "transformed cell" as used herein, includes the progeny of originally transformed cells.

Techniques for manipulation of nucleic acids which encode the polypeptides of the present invention, i.e., subcloning the nucleic acids into expression vectors, labeling probes, DNA hybridization and the like, are generally described in Sambrook, et al., supra. In recombinant methods, generally the nucleic acid encoding a peptide of the present invention is first cloned or isolated in a form suitable for ligation into an expression vector. After ligation, the vectors containing the nucleic acids fragments or inserts are introduced into a suitable host cell, for the expression of the polypeptide of the invention. The polypeptides may then be purified or isolated from the host cells. Methods for the synthetic preparation of oligonucleotides are generally described in Gait, oligonucleotide Synthesis: A Practical Approach, IRL Press (1990).

There are various methods of isolating the nucleic acids which encode the polypeptides of the present invention. Typically, the DNA is isolated from a genomic or cDNA library using labeled oligonucleotide probes specific for sequences in the desired DNA. Restriction endonuclease digestion of genomic DNA or cDNA containing the appropriate genes can be used to isolate the DNA encoding the binding domains of these proteins. From the PfEMP1 sequence given (as shown herein), a panel of restriction endonucleases can be constructed to give cleavage of the DNA in desired regions, i.e., to obtain segments which encode biologically active fragments of the PfEMP1 protein.

Following restriction endonuclease digestion, DNA encoding the polypeptides of the present invention is identified by its ability to hybridize with a nucleic acid probe in, for example a Southern blot format. These regions are then isolated using standard methods. See, e.g., Sambrook, et al., *supra*.

5 The polymerase chain reaction, or "PCR" can also be used to prepare nucleic acids which encode the polypeptides of the present invention. PCR technology is used to amplify nucleic acid sequences of the desired nucleic acid, e.g., the DNA which encodes the polypeptides of the invention, directly from mRNA, cDNA, or genomic or cDNA libraries.

10 Appropriate primers and probes for amplifying the nucleic acids described herein, may be generated from analysis of the PfEMP1 oligonucleotide sequence, such as those shown, described &/or referenced herein (including incorporated by reference) and Table 1. Briefly, oligonucleotide primers complementary to the two 31 borders of the DNA region to be amplified are synthesized. The PCR is then carried out using the two primers. See, e.g., PCR Protocols: A Guide to Methods and Applications (Innis, M., Gelfand, D., Sninsky, J. and
15 White, T., eds.) Academic Press (1990). Primers can be selected to amplify various sized segments from the PfEMP1 oligonucleotide sequence. The primers may also contain a restriction site and additional bases to permit "in-frame" cloning of the insert into an appropriate expression vector, using the restriction sites present on the primers.

Antibodies

20 The nucleic acids and polypeptides of the present invention, or fragments thereof, are also useful in producing antibodies, either polyclonal or monoclonal. These antibodies are produced by immunizing an appropriate vertebrate host, e.g., rat, mouse, rabbit or goat, with a polypeptide of the invention, or its fragment, or plasmid DNA containing a nucleic acid of the invention, alone or in conjunction with an adjunct. Usually, two or more immunizations
25 are involved, and a few days following the last injection, the blood or spleen of the host will be harvested.

30 For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit, but also including goats, sheep, cows, guinea pigs, monkeys and rats. The substantially purified antigen or plasmid is presented to the immune system in a fashion determined by methods appropriate for the animal. These and other parameters are well known to immunologists. Typically, injections are given in the footpads, intramuscularly, intradermally or intraperitoneally. The immunoglobulins produced by the

host can be precipitated, isolated and purified by routine methods, including affinity purification.

For monoclonal antibodies, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of these animals are excised and individual spleen cells are fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone are tested for the production of an appropriate antibody specific for the desired region of the antigen. Techniques for producing antibodies are well known in the art. See, e.g., Goding et al., *Monoclonal Antibodies: Principles and Practice* (2d ed.) Acad. Press, N.Y., and Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988). Other suitable techniques involve the *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively, to selection of libraries of antibodies in phage or similar vectors. Huse et al., *Generation of Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda*, *Science* 246:1275-1281 (1989). Monoclonal antibodies with affinities of 10^8 liters/mole, preferably 10^9 to 10^{10} or stronger, will be produced by these methods.

The antibodies generated can be used for a number of purposes, e.g., as probes in immunoassays, for inhibiting PfEMP1 binding to its ligands, thereby inhibiting or reducing erythrocyte sequestration, in diagnostics or therapeutics, or in research to further elucidate the mechanism of various aspects of malarial infection, and particularly, *P. falciparum* infection. The antibodies of the present invention can be used with or without modification. Frequently, the antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. Such labels include those that are well known in the art, such as the labels described previously for the polypeptides of the invention. Additionally, the antibodies of the invention may be chimeric, human-like or humanized, in order to reduce their potential antigenicity, without reducing their affinity for their target. Chimeric, human-like and humanized antibodies have generally been described in the art. Generally, such chimeric, human-like or humanized antibodies comprise variable regions, e.g., complementarity determining regions (CDR) (for humanized antibodies), from a mammalian animal, i.e., a mouse, and a human framework region. By incorporating as little foreign sequence as possible in the hybrid antibody, the antigenicity is reduced. Preparation of these hybrid antibodies may be carried out by methods well known in the art.

Preferred antibodies are those that are specifically immunoreactive with the polypeptides of the present invention and their immunologically active fragments. The phrase "specifically immunoreactive," when referring to the interaction between an antibody of the invention and a particular protein, refers to an antibody that specifically recognizes and binds with relatively high affinity to the particular protein, such that this binding is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The antibodies generated can be used for a number of purposes, e.g., as probes in immunoassays, for inhibiting interaction between a PfEMP1 protein and its ligand, e.g., CD-36, TSP, ICAM-1, VCAM-1, ELAM-1, or Chondroitin sulfate, thereby inhibiting or reducing the level of PfEMP1-ligand interaction, in diagnostics or therapeutics, or in research to further elucidate the mechanism of malarial pathology, e.g., erythrocyte sequestration. Where the antibodies are used to block or reverse the interaction between a polypeptide of the invention and an associating ligand or PE, the antibody will generally be referred to as a "blocking antibody." Preferred antibodies are those monoclonal or polyclonal antibodies which specifically recognize and bind the polypeptides of the invention. Accordingly, these preferred antibodies will specifically recognize and bind the polypeptides which have an amino acid sequence that is substantially homologous to the relevant amino acid sequence shown, described &/or referenced herein (including incorporated by reference), or immunologically active fragments thereof. Still more preferred are antibodies which are capable of forming an antibody-ligand complex with the relatively conserved polypeptide fragments of PfEMP1 sequences, and are thereby capable of blocking an interaction of PfEMP1 from a variety of *P. falciparum* strains, and PfEMP1 ligands.

METHODS OF USE

The polypeptides, antibodies, and nucleic acids of the present invention have a variety of important uses, including, but not limited to, diagnostic, screening, prophylactic, including vaccination, and therapeutic applications.

Diagnostic Applications

5 In a particularly preferred aspect, the present invention provides methods and reagents useful in detecting the presence of PfEMP1 in a sample. These detection methods are particularly useful in diagnosing malarial infections in a patient. For example, in a particularly preferred aspect, the antibodies of the present invention may be used to assay for the presence or absence of PfEMP1 in a sample. Immunoassay techniques for the detection
10 of the particular antigen are very well known in the art. For a review of immunological and immunoassay procedures in general, see Basic and Clinical Immunology 7th Edition (D. Stites and A. Terr ed.) 1991.

Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); "Practice and Theory of Enzyme
15 Immunoassays," P. Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers B.V. Amsterdam (1985); and, Harlow and Lane, Antibodies, A Laboratory Manual, supra. Generally, these methods comprise contacting the antibody with a sample to be tested, and detecting any specific binding between the antibody and a protein
20 within the sample. Typically, this will be in a blot format, e.g., western blot, or in an ELISA format. Methods of performing these assay formats are well known in the art. See, e.g., Basic and Clinical Immunology, 7th ed. (D. Stites and A. Terr, eds., 1991).

Typically, these diagnostic methods comprise contacting a sample with an antibody to PfEMP1, as described herein, and determining whether the antibody binds to any portion of
25 the sample. In the case of human diagnostic techniques, the sample may be a whole blood sample, or some fraction thereof, e.g. an erythrocyte containing sample. Generally, such diagnostic methods are well known in the art, and are described in the above described references. The immunoreactivity of the antibody with the sample, indicates the presence of PfEMP1 in the sample, and, in the case of a sample derived from a patient, a possible
30 malarial infection.

Alternatively, labeled polypeptides of the present invention may be used as diagnostic reagents in detecting the presence or absence of antibodies to PfEMP1, in a patient. The

presence of antibodies within a patient would be indicative that the patient had been exposed to a malaria parasite sufficiently to result in an antigenic response.

Similarly, the nucleic acid probes of the invention may be used in a similar manner, i.e., to identify the presence in a sample of a DNA segment encoding a PfEMP1 polypeptide, or as PCR or RT-PCR primers to amplify and then detect PfEMP1 encoding nucleic acid segments. Such assays typically involve the immobilization of nucleic acids in the sample, followed by interrogation?? of the immobilized sequences with a chemically labeled oligonucleotide probe, as described herein. Hybridization of the probe to the immobilized sample indicates the presence of a DNA segment encoding PfEMP1, and thus, a malarial infection. As described above, assays may be further designed to indicate not only the presence of a Malarial parasite, but also indicate the strain of parasite present. Although described in terms of an immobilized sample probed with a solution based oligonucleotide probe, a wide variety of assay conformations may be adopted, which conformations are generally well known in the art.

Screening Applications

In one aspect, the present invention provides methods for screening compounds to determine whether or not the particular compound is an antagonist of a symptom of a malarial infection. In particular, the screening methods of the present invention can be used to determine whether a test compound is an antagonist of the sequestration of erythrocytes which is associated with *P. falciparum* malaria. More particularly, the screening methods can determine whether a compound is an antagonist of the PfEMP1/ligand interaction. Ligands of PfEMP1 generally include, e.g., CD36, TSP, ELAM-1, ICAM-1, VCAM-1 or Chondroitin sulfate.

Generally, the screening methods of the present invention comprise contacting PfEMP1 protein, or a fragment thereof, and/or ligand protein, with a compound which is to be screened ("test compound"). The level of PfEMP1/ligand complex formed may then be detected and compared to a control, e.g., in the absence of the test compound. A decrease in the level of PfEMP1/ligand interaction is indicative that the test compound is an antagonist of that interaction.

A test compound may be a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials, such as bacteria, phage, yeast, plants, fungi, animal cells or tissues. Test compounds are evaluated for potential

activity as antagonists of PfEMP1/ligand interaction by inclusion in the screening assays described herein. An "antagonist" refers to a compound which will diminish the level of PfEMP1/ligand interaction, over a control.

It will often be desirable in the screening assays of the present invention, to provide one of the PfEMP1 or ligand proteins immobilized on a solid support. Suitable solid supports include, e.g., agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose, polystyrene, filter paper, nitrocellulose, ion exchange resins, plastic films, glass beads, polyaminemethylvinylether maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The support may be in the form of, e.g., a test tube, microtiter plate, beads, test strips, flat surface, e.g., for blotting formats, or the like. The reaction of the PfEMP1 polypeptide or its ligand with the particular solid support may be carried out by methods well known in the art, e.g., binding to an immobilized anti-PfEMP1 antibody, or binding to prederivatized solid support.

In addition to the foregoing, it may also be desirable to provide either the PfEMP1 or its ligand linked to a suitable detectable group to make detection of binding of one protein to the other, simpler. Useful detectable groups, or labels, are generally well known in the art. For example, a detectable group may be a radiolabel, such as, ^{125}I , ^{32}P or ^{35}S , or a fluorescent or chemiluminescent group.

Alternatively, the detectable group may be a substrate, cofactor, inhibitor, affinity ligand, antibody binding epitope tag, or an enzyme which is capable of being assayed. Suitable enzymes include, e.g., horseradish peroxidase, luciferase, or another readily assayable enzymes. These enzyme groups may be attached to the PfEMP1 polypeptide, or its ligand by chemical means or maybe expressed as a fusion protein, as already described.

Generally, where one of the above proteins, e.g., the PfEMP1 ligand, is immobilized on a solid support, the other protein, e.g., PfEMP1 or its fragment, will be labeled with an appropriate detectable group. Assaying whether a compound is an antagonist of the interaction of the two proteins is then a matter of contacting the labeled PfEMP1 polypeptide or fragment with the immobilized ligand, in the presence of the test compound, under conditions which allow specific binding of the two proteins. The amount of label bound to the solid support is compared to a control, where no test compound was added. Where a test compound results in a reduction of the amount of label which binds to a solid support, that compound is an antagonist of the PfEMP1/ligand interaction.

Therapeutic And Prophylactic Applications

In addition to the above described uses, the polypeptides of the present invention may also be used in therapeutic applications, for the treatment of human and/or non-human mammalian patients. The therapeutic uses of the polypeptides of the present invention include the treatment of symptoms of existing disorders, as well as prophylactic applications. The term "prophylactic" refers to the prevention of a particular disorder, or symptoms of a particular disorder. Thus, prophylactic treatments will generally include drugs which actively participate in the prevention of a particular disorder such as a malaria infection, or symptoms thereof. Prophylactic applications will also include treatments which elicit a preventative response from a patient, including, for example, an immunological response as in the case of vaccination.

Typically, both therapeutic and prophylactic applications will comprise administering an effective amount of the compositions of the present invention to a patient, to treat or prevent symptoms, or the onset of a malarial parasite infection. An "effective amount", as the term is used herein, is defined as the amount of the composition which is necessary to achieve the desired goal, i.e. alleviation of symptoms, prevention of symptoms or infection, or treatment of disease.

In prophylactic applications, the polypeptides of the present invention may be used in a variety of treatments. For example, the polypeptides of the invention are particularly useful as a vaccine, to elicit an immunological response by a patient, e.g., production of antibodies specific for PfEMP1. In particular, such vaccine applications generally involve the administration of the PfEMP1 protein or biologically active fragments thereof, to the host or patient.

In response to this administration, the patient's immune system will generate antibodies to the particular PfEMP1 protein or fragment introduced. An amount of the polypeptides sufficient to produce an immunological response in a patient is termed "an immunogenically effective amount." Thus, the vaccines of the present invention will contain an immunogenically effective amount of the polypeptides of the present invention. The immune response of the patient may include generation of antibodies, activation of cytotoxic T- lymphocytes against cells expressing the polypeptides, e.g., PE, or other mechanisms known to the skilled artisan. See, e.g., Paul, Fundamental Immunology, 2d Edition, Raven

Press. Useful carriers are well known in the art, and include for example, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine; D- glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine. The vaccines can also contain a physiologically tolerable diluent, such as water, buffered water, buffered saline, saline and typically may further include an adjuvant, such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, alum, or other materials well known in the art.

Alternatively, the nucleic acids of the present invention may also be used as vaccines for the prevention of malaria symptoms, and/or infection by malaria parasites. See Sedegah, et al. Proc. Nat'l Acad. Sci. (1994) 91:9866-9870.

For example, plasmid DNA comprising the nucleic acids of the present invention may be directly administered to a patient. Expression of this "naked" DNA will have effects similar to the injection of the actual polypeptides, as described above. Specifically, the patient's immune response to the presence of the proteins expressed from the DNA, will result in the production of antibodies to that protein. The nucleic acids may also be used to design antisense probes to interrupt transcription of PfEMP1 peptides in parasitized erythrocytes.

Antisense methods are generally well known in the art. The polypeptides of the present invention, and analogs thereof, may also be used as prophylactic treatments to prevent the onset of symptoms of malarial infection. For example, administration of the polypeptides can directly inhibit, block or reverse the sequestration of erythrocytes in patients suffering from *P. falciparum* malaria infections. In particular, the polypeptides of the invention may be used to compete with or displace PE associated PfEMP1 in binding CD36.

The blockage or reversal of sequestration will reduce or eliminate the microvascular occlusion generally associated with the pathology of this type of malaria, which, again, can lead to destruction of the PE by the host. The antibodies of the invention may also be used in a similar fashion. In particular, the antibodies, which are capable of binding the polypeptides of the present invention, may be directly administered to a patient. By binding PfEMP1, the antibodies of the present invention are effective in blocking, reducing or reversing PfEMP1 mediated interactions, e.g., erythrocyte sequestration. Chimeric, human-like or humanized antibodies are particularly useful for administration to human patients. Additionally, such

antibodies may also be used as a passive vaccination method to provide a subject with a short term immunization, much as anti-hepatitis A injections have been used previously.

In alternative aspects, the polypeptides, antibodies and nucleic acids of the invention may be used to treat a patient already suffering from a malarial infection. In particular, the compositions of the present invention may be administered to a patient suffering from a malarial infection to treat symptoms associated with that infection. More particularly, these compositions may be administered to the patient to prevent or reduce erythrocyte sequestration and the resulting microvascular occlusion associated with malarial, and more specifically, *P. falciparum*, infections.

Although the polypeptides, nucleic acids and antibodies of the present invention may be administered alone, for therapeutic and prophylactic applications, these elements will generally be administered as part of a pharmaceutical composition, e.g., in combination with a pharmaceutically acceptable carrier. Typically, a single composition may be used in both therapeutic and prophylactic applications. Pharmaceutical formulations suitable for use in the present invention are generally described in Remington's Pharmaceutical Sciences, Mack Publishing Co., 17th ed. (1985).

The pharmaceutical compositions of the present invention are intended for parenteral, topical, oral, or local administration. Where the pharmaceutical compositions are administered parenterally, the invention provides pharmaceutical compositions that comprise a solution of the agents described above, e.g., polypeptides of the invention, dissolved or suspended in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, saline glycine, and the like. These compositions may be sterilized by conventional, well known methods, e.g., sterile filtration. The resulting aqueous solutions may be packaged for use as is, or lyophilized for combination with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, and the like, for example sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For oral administration, a pharmaceutically acceptable nontoxic composition may be formed by incorporating any of the normally employed excipients, such as the previously listed carriers, and generally, 10-95% of active ingredient, and more preferably 25-75% active ingredient. In addition, for oral administration of peptide based compounds, the pharmaceutical compositions may include the active ingredient as part of a matrix to prevent proteolytic degradation of the active ingredient by digestive process, e.g., by providing the pharmaceutical composition within a liposomal composition, according to methods well known in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Co., 17th Ed. (1985).

For aerosol administration, the polypeptides are generally supplied in finely divided form along with a surfactant or propellant. Preferably, the surfactant will be soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids, with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery. The above described compositions are suitable for a single administration or a series of administrations. When given as a series, e.g., as a vaccine booster, the inoculations subsequent to the initial administration are given to boost the immune response, and are typically referred to as booster inoculations.

The amount of the above compositions to be administered to the patient will vary depending upon what is to be administered to the patient, the state of the patient, the manner of administration, and the particular application, e.g., therapeutic or prophylactic. In therapeutic applications, the compositions are administered to the patient already suffering from a malarial infection, in an amount sufficient to inhibit the spread of the parasite through the erythrocytes, and thereby cure or at least partially arrest the symptoms of the disease and its associated complications.

An amount adequate to accomplish this is termed "a therapeutically effective amount." Amounts effective for this use will depend upon the severity of the disease and the weight and general state of the patient, but will generally be in the range of from about 1 mg to about 5 g of active agent per day, preferably from about 50 mg per day to about 500 mg per day, and more preferably, from about 50 mg to about 100 mg per day, for a 70 kg patient.

For prophylactic applications, immunogenically effective amounts will also depend upon the composition, the manner of administration and the weight and general state of the patient, as well as the judgment of the prescribing physician. For the peptide, peptide analog and antibody based pharmaceutical compositions, the general range for the initial immunization (for either prophylactic or therapeutic applications) will be from about 100 μ g to about 1 g of polypeptide for a 70 kg patient, followed by boosting dosages of from about 1 μ g to about 1 gm of polypeptide pursuant to a boosting regimen over weeks to months, depending upon the patient's response and condition, e.g., by measuring the level of parasite or antibodies in the patient's blood. For nucleic acids, typically from about 30 to about 100 μ g of nucleic acid is injected into a 70 kg patient, more typically, about 50 to 150 μ g of nucleic acid is injected, followed by boosting treatments as appropriate.

DIRECTED EVOLUTION METHODS

In one aspect the invention described herein is directed to the use of repeated cycles of reductive reassortment, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences, such as DNA, RNA or proteins thorough recombination.

In vivo shuffling of molecules can be performed utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the chiasma requires the recognition of homologous sequences.

In a preferred embodiment, the invention relates to a method for producing a hybrid polynucleotide from at least a first polynucleotide and a second polynucleotide. The present invention can be used to produce a hybrid polynucleotide by introducing at least a first polynucleotide and a second polynucleotide which share at least one region of partial sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present invention and contains sequence from

at least two original polynucleotide sequences. Such hybrid polynucleotides can result from intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

The invention provides a means for generating hybrid polynucleotides which may encode biologically active hybrid polypeptides. In one aspect, the original polynucleotides encode biologically active polypeptides. The method of the invention produces new hybrid polypeptides by utilizing cellular processes which integrate the sequence of the original polynucleotides such that the resulting hybrid polynucleotide encodes a polypeptide demonstrating activities derived from the original biologically active polypeptides. For example, the original polynucleotides may encode a particular enzyme from different microorganisms. An enzyme encoded by a first polynucleotide from one organism may, for example, function effectively under a particular environmental condition, e.g. high salinity. An enzyme encoded by a second polynucleotide from a different organism may function effectively under a different environmental condition, such as extremely high temperatures. A hybrid polynucleotide containing sequences from the first and second original polynucleotides may encode an enzyme which exhibits characteristics of both enzymes encoded by the original polynucleotides. Thus, the enzyme encoded by the hybrid polynucleotide may function effectively under environmental conditions shared by each of the enzymes encoded by the first and second polynucleotides, e.g., high salinity and extreme temperatures.

Enzymes encoded by the original polynucleotides of the invention include, but are not limited to; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. A hybrid polypeptide resulting from the method of the invention may exhibit specialized enzyme activity not displayed in the original enzymes. For example, following recombination and/or reductive reassortment of polynucleotides encoding hydrolase activities, the resulting hybrid polypeptide encoded by a hybrid polynucleotide can be screened for specialized hydrolase activities obtained from each of the original enzymes, i.e. the type of bond on which the hydrolase acts and the temperature at which the hydrolase functions. Thus, for example, the hydrolase may be screened to ascertain those chemical functionalities which distinguish the hybrid hydrolase from the original hydrolyases, such as:

(a) amide (peptide bonds), i.e. proteases; (b) ester bonds, i.e. esterases and lipases; (c) acetals, i.e., glycosidases and, for example, the temperature, pH or salt concentration at which the hybrid polypeptide functions.

Sources of the original polynucleotides may be isolated from individual organisms ("isolates"), collections of organisms that have been grown in defined media ("enrichment cultures"), or, most preferably, uncultivated organisms ("environmental samples"). The use of a culture-independent approach to derive polynucleotides encoding novel bioactivities from environmental samples is most preferable since it allows one to access untapped resources of biodiversity.

"Environmental libraries" are generated from environmental samples and represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

For example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries. Polynucleotides encoding activities of interest are isolated from such libraries and introduced into a host cell. The host cell is grown under conditions which promote recombination and/or reductive reassortment creating potentially active biomolecules with novel or enhanced activities.

The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as Eubacteria and Archaeobacteria, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect, such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes

isolated from extremophilic microorganisms are particularly preferred. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge. For example, several esterases and lipases cloned and expressed from extremophilic organisms show high activity throughout a wide range of temperatures and pHs.

Polynucleotides selected and isolated as hereinabove described are introduced into a suitable host cell. A suitable host cell is any cell which is capable of promoting recombination and/or reductive reassortment. The selected polynucleotides are preferably already in a vector which includes appropriate control sequences. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or preferably, the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis et al, 1986).

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

With particular references to various mammalian cell culture systems that can be employed to express recombinant protein, examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described in "SV40-transformed simian cells support the replication of early SV40 mutants" (Gluzman, 1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Host cells containing the polynucleotides of interest can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the
5 ordinarily skilled artisan. The clones which are identified as having the specified enzyme activity may then be sequenced to identify the polynucleotide sequence encoding an enzyme having the enhanced activity.

In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or
10 gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene
15 cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many
20 polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating
25 genetic manipulation and *in vitro* studies of these genes/proteins.

The ability to select and combine desired components from a library of polyketides, or fragments thereof, and postpolyketide biosynthesis genes for generation of novel polyketides for study is appealing. The method of the present invention makes it possible to facilitate the production of novel polyketide synthases through intermolecular recombination.

30 Preferably, gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity

from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

Therefore, in a preferred embodiment, the present invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

- 1) introducing at least a first polynucleotide in operable linkage and a second polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
- 2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
- 3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
- 4) screening the hybrid polypeptide under conditions which promote identification of enhanced biological activity; and
- 5) isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the art and discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the present invention.

As representative examples of expression vectors which may be used there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (e.g. vaccinia, adenovirus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids,

yeast artificial chromosomes, and any other vectors specific for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the DNA may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences. Large numbers of suitable
5 vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid or other vector may be used as long as
10 they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

A preferred type of vector for use in the present invention contains an f-factor origin replication. The f-factor (or fertility factor) in *E. coli* is a plasmid which effects high frequency transfer of itself during conjugation and less frequent transfer of the bacterial
15 chromosome itself. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library."

20 Another preferred type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in "Molecular Cloning: A laboratory Manual" (Sambrook et al, 1989).

The DNA sequence in the expression vector is operatively linked to an appropriate
25 expression control sequence(s) (promoter) to direct RNA synthesis. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also
30 contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Promoter regions

can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium.

The cloning strategy permits expression via both vector driven and endogenous promoters; vector promotion may be important with expression of genes whose endogenous promoter will not function in *E. coli*.

The DNA isolated or derived from microorganisms can preferably be inserted into a vector or a plasmid prior to probing for selected DNA. Such vectors or plasmids are preferably those containing expression regulatory sequences, including promoters, enhancers and the like. Such polynucleotides can be part of a vector and/or a composition and still be isolated, in that such vector or composition is not part of its natural environment. Particularly preferred phage or plasmid and methods for introduction and packaging into them are described in detail in the protocol set forth herein.

The selection of the cloning vector depends upon the approach taken, for example, the vector can be any cloning vector with an adequate capacity for multiply repeated copies of a sequence, or multiple sequences that can be successfully transformed and selected in a host cell. One example of such a vector is described in "Polycos vectors: a system for packaging filamentous phage and phagemid vectors using lambda phage packaging extracts" (Alting-Mecs and Short, 1993). Propagation/maintenance can be by an antibiotic resistance

carried by the cloning vector. After a period of growth, the naturally abbreviated molecules are recovered and identified by size fractionation on a gel or column, or amplified directly. The cloning vector utilized may contain a selectable gene that is disrupted by the insertion of the lengthy construct. As reductive reassortment progresses, the number of repeated units is reduced and the interrupted gene is again expressed and hence selection for the processed construct can be applied. The vector may be an expression/selection vector which will allow for the selection of an expressed product possessing desirable biological properties. The insert may be positioned downstream of a functional promoter and the desirable property screened by appropriate means.

In vivo reassortment is focused on "inter-molecular" processes collectively referred to as "recombination" which in bacteria, is generally viewed as a "RecA-dependent" phenomenon. The present invention can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells' ability to mediate reductive processes to decrease the complexity of quasi-repeated sequences in the cell by deletion. This process of "reductive reassortment" occurs by an "intra-molecular", RecA-independent process.

Therefore, in another aspect of the present invention, novel polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their insertion into an appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel molecular species. Various treatments may be applied to enhance the rate of reassortment. These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive

sequences become essentially invisible and the quasi-repetitive nature of the resulting construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion) events can occur virtually anywhere within the quasi-repetitive units.

When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases the efficiency, it may still provide sufficient elasticity for the effective recovery of novel molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

- a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNaseH.
- b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.
- c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced RI. The re-assorted encoding sequences can then be recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be effected by:

- 1) The use of vectors only stably maintained when the construct is reduced in complexity.
- 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.
- 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.
- 4) The use of direct selection techniques with an expression vector and the appropriate selection.

Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

The following example demonstrates the method of the invention. Encoding nucleic acid sequences (quasi-repeats) derived from three (3) unique species are depicted. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence which are designated "A", "B" and "C". The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an

appropriate host cell. The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. Whether the reduction in RI is mediated by deletion formation between repeated sequences by an "intra-molecular" mechanism, or mediated by recombination-like events through "inter-molecular" mechanisms is immaterial. The end result is a reassortment of the molecules into all possible combinations.

Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact (e.g., such as catalytic antibodies) with a predetermined macromolecule, such as for example a proteinaceous receptor, peptide oligosaccharide, viron, or other predetermined compound or structure.

The displayed polypeptides, antibodies, peptidomimetic antibodies, and variable region sequences that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (e.g., catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or affinity selection. The method can be modified such that the step of selecting for a phenotypic characteristic can be other than of binding affinity for a predetermined molecule (e.g., for catalytic activity, stability oxidation resistance, drug resistance, or detectable phenotype conferred upon a host cell).

The present invention provides a method for generating libraries of displayed antibodies suitable for affinity interactions screening. The method comprises (1) obtaining first a plurality of selected library members comprising a displayed antibody and an associated polynucleotide encoding said displayed antibody, and obtaining said associated polynucleotide encoding for said displayed antibody and obtaining said associated polynucleotides or copies thereof, wherein said associated polynucleotides comprise a region of substantially identical variable region framework sequence, and (2) introducing said polynucleotides into a suitable host cell and growing the cells under conditions which promote recombination and reductive reassortment resulting in shuffled polynucleotides. CDR combinations comprised by the shuffled pool are not present in the first plurality of selected library members, said shuffled pool composing a library of displayed antibodies comprising CDR permutations and suitable for affinity interaction screening. Optionally, the shuffled pool is subjected to affinity screening to select shuffled library members which bind

to a predetermined epitope (antigen) and thereby selecting a plurality of selected shuffled library members. Further, the plurality of selectively shuffled library members can be shuffled and screened iteratively, from 1 to about 1000 cycles or as desired until library members having a desired binding affinity are obtained.

5 In another aspect of the invention, it is envisioned that prior to or during recombination or reassortment, polynucleotides generated by the method of the present invention can be subjected to agents or processes which promote the introduction of mutations into the original polynucleotides. The introduction of such mutations would increase the diversity of resulting hybrid polynucleotides and polypeptides encoded therefrom. The agents or processes which promote mutagenesis can include, but are not limited to: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine, see Sun and Hurley, 1992); an N-acetylated or deacetylated 4'-fluoro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see, for example, van de Poll et al, 1992); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (see also, van de Poll et al, 1992, pp. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon ("PAH") DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[*a*]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[*a*]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-*f*]-quinoline ("N-hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*f*]-pyridine ("N-hydroxy-PhIP"). Especially preferred "means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotides pool, which can be released or removed by a process including heating the solution comprising the polynucleotides prior to further processing.

In another aspect the present invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under conditions according to the present invention which provide for the production of hybrid or re-assorted polynucleotides.

The invention also provides the use of polynucleotide shuffling to shuffle a population of viral genes (e.g., capsid proteins, spike glycoproteins, polymerases, and proteases) or viral genomes (e.g., paramyxoviridae, orthomyxoviridae, herpesviruses, retroviruses, reoviruses and rhinoviruses). In an embodiment, the invention provides a method for shuffling sequences encoding all or portions of immunogenic viral proteins to generate novel combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes as well as novel epitopes created by recombination; such shuffled viral proteins may comprise epitopes or combinations of epitopes which are likely to arise in the natural environment as a consequence of viral evolution; (e.g., such as recombination of influenza virus strains).

The invention also provides a method suitable for shuffling polynucleotide sequences for generating gene therapy vectors and replication-defective gene therapy constructs, such as may be used for human gene therapy, including but not limited to vaccination vectors for DNA-based vaccination, as well as anti-neoplastic gene therapy and other general therapy formats.

In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences".

Saturation Mutagenesis

In one aspect, this invention provides for the use of proprietary codon primers (containing a degenerate N,N,G/T sequence) to introduce point mutations into a polynucleotide, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position. The oligos used are comprised contiguously of a first homologous sequence, a degenerate N,N,G/T sequence, and preferably but not necessarily a second homologous sequence. The downstream progeny

translational products from the use of such oligos include all possible amino acid changes at each amino acid site along the polypeptide, because the degeneracy of the N,N,G/T sequence includes codons for all 20 amino acids.

5 In one aspect, one such degenerate oligo (comprised of one degenerate N,N,G/T cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,G/T cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. Thus, more than one N,N,G/T sequence can be contained in one oligo to introduce amino acid mutations at
10 more than one site. This plurality of N,N,G/T sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,G/T sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

15 In a particular exemplification, it is possible to simultaneously mutagenize two or more contiguous amino acid positions using an oligo that contains contiguous N,N,G/T triplets, i.e. a degenerate (N,N,G/T)_n sequence.

In another aspect, the present invention provides for the use of degenerate cassettes having less degeneracy than the N,N,G/T sequence. For example, it may be desirable in
20 some instances to use (e.g. in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (e.g. in an oligo) a degenerate N,N,N triplet sequence, or an N,N, G/C triplet sequence.

25 It is appreciated, however, that the use of a degenerate triplet (such as N,N,G/T or an N,N, G/C triplet sequence) as disclosed in the instant invention is advantageous for several reasons. In one aspect, this invention provides a means to systematically and fairly easily generate the substitution of the full range of possible amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide. Thus, for a 100 amino acid
30 polypeptide, the instant invention provides a way to systematically and fairly easily generate 2000 distinct species (i.e. 20 possible amino acids per position X 100 amino acid positions). It is appreciated that there is provided, through the use of an oligo containing a degenerate

N,N,G/T or an N,N, G/C triplet sequence, 32 individual sequences that code for 20 possible amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using one such oligo, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligo in site-directed mutagenesis leads to only one progeny polypeptide product per reaction vessel.

This invention also provides for the use of nondegenerate oligos, which can optionally be used in combination with degenerate primers disclosed. It is appreciated that in some situations, it is advantageous to use nondegenerate oligos to generate specific point mutations in a working polynucleotide. This provides a means to generate specific silent point mutations, point mutations leading to corresponding amino acid changes, and point mutations that cause the generation of stop codons and the corresponding expression of polypeptide fragments.

Thus, in a preferred embodiment of this invention, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide molecules such that all 20 amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide. The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel can be subjected to clonal amplification (e.g. cloned into a suitable *E. coli* host using an expression vector) and subjected to expression screening. When an individual progeny polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide), it can be sequenced to identify the correspondingly favorable amino acid substitution contained therein.

It is appreciated that upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid changes may be identified at more than one amino acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if 2 specific favorable amino acid changes are identified in each of 3 amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes) and 3 positions. Thus, there are $3 \times 3 \times 3$ or 27 total possibilities, including 7 that were

previously examined - 6 single point mutations (i.e. 2 at each of three positions) and no change at any position.

In yet another aspect, site-saturation mutagenesis can be used together with shuffling, chimerization, recombination and other mutagenizing processes, along with screening. This invention provides for the use of any mutagenizing process(es), including saturation mutagenesis, in an iterative manner. In one exemplification, the iterative use of any mutagenizing process(es) is used in combination with screening.

Thus, in a non-limiting exemplification, this invention provides for the use of saturation mutagenesis in combination with additional mutagenization processes, such as process where two or more related polynucleotides are introduced into a suitable host cell such that a hybrid polynucleotide is generated by recombination and reductive reassortment.

In addition to performing mutagenesis along the entire sequence of a gene, the instant invention provides that mutagenesis can be used to replace each of any number of bases in a polynucleotide sequence, wherein the number of bases to be mutagenized is preferably every integer from 15 to 100,000. Thus, instead of mutagenizing every position along a molecule, one can subject every a discrete number of bases (preferably a subset totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer, containing a heterologous cassette, also referred to as a mutagenic cassette. Preferred cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, C/G/T, A/G/T, A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo). The tables below show exemplary tri-nucleotide cassettes (there are over 3000 possibilities in addition to N,N,G/T and N,N,N and N,N,A/C).

In a general sense, saturation mutagenesis is comprised of mutagenizing a complete set of mutagenic cassettes (wherein each cassette is preferably 1-500 bases in length) in defined polynucleotide sequence to be mutagenized (wherein the sequence to be mutagenized is preferably from 15 to 100,000 bases in length). Thusly, a group of mutations (ranging from 1 to 100 mutations) is introduced into each cassette to be mutagenized. A grouping of mutations to be introduced into one cassette can be different or the same from a second grouping of mutations to be introduced into a second cassette during the application of one

round of saturation mutagenesis. Such groupings are exemplified by deletions, additions, groupings of particular codons, and groupings of particular nucleotide cassettes.

Defined sequences to be mutagenized (see Fig. 20) include preferably a whole gene, pathway, cDNA, an entire open reading frame (ORF), and entire promoter, enhancer, repressor/transactivator, origin of replication, intron, operator, or any polynucleotide functional group. Generally, a preferred "defined sequences" for this purpose may be any polynucleotide that a 15 base-polynucleotide sequence, and polynucleotide sequences of lengths between 15 bases and 15,000 bases (this invention specifically names every integer in between). Considerations in choosing groupings of codons include types of amino acids encoded by a degenerate mutagenic cassette.

In a particularly preferred exemplification a grouping of mutations that can be introduced into a mutagenic cassette (see Tables 1-85), this invention specifically provides for degenerate codon substitutions (using degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 amino acids at each position, and a library of polypeptides encoded thereby.

Chimerizations

"Shuffling"

Nucleic acid shuffling is a method for *in vitro* or *in vivo* homologous recombination of pools of shorter or smaller polynucleotides to produce a polynucleotide or polynucleotides. Mixtures of related nucleic acid sequences or polynucleotides are subjected to sexual PCR to provide random polynucleotides, and reassembled to yield a library or mixed population of recombinant hybrid nucleic acid molecules or polynucleotides.

In contrast to cassette mutagenesis, only shuffling and error-prone PCR allow one to mutate a pool of sequences blindly (without sequence information other than primers).

The advantage of the mutagenic shuffling of this invention over error-prone PCR alone for repeated selection can best be explained with an example from antibody engineering. Consider DNA shuffling as compared with error-prone PCR (not sexual PCR). The initial library of selected pooled sequences can consist of related sequences of diverse origin (i.e. antibodies from naive mRNA) or can be derived by any type of mutagenesis (including shuffling) of a single antibody gene. A collection of selected complementarity determining regions ("CDRs") is obtained after the first round of affinity selection. In the

diagram the thick CDRs confer onto the antibody molecule increased affinity for the antigen. Shuffling allows the free combinatorial association of all of the CDR1s with all of the CDR2s with all of the CDR3s, for example.

This method differs from error-prone PCR, in that it is an inverse chain reaction. In error-prone PCR, the number of polymerase start sites and the number of molecules grows exponentially. However, the sequence of the polymerase start sites and the sequence of the molecules remains essentially the same. In contrast, in nucleic acid reassembly or shuffling of random polynucleotides the number of start sites and the number (but not size) of the random polynucleotides decreases over time. For polynucleotides derived from whole plasmids the theoretical endpoint is a single, large concatemeric molecule.

Since cross-overs occur at regions of homology, recombination will primarily occur between members of the same sequence family. This discourages combinations of CDRs that are grossly incompatible (e.g., directed against different epitopes of the same antigen). It is contemplated that multiple families of sequences can be shuffled in the same reaction. Further, shuffling generally conserves the relative order, such that, for example, CDR1 will not be found in the position of CDR2.

Rare shufflants will contain a large number of the best (eg. highest affinity) CDRs and these rare shufflants may be selected based on their superior affinity.

CDRs from a pool of 100 different selected antibody sequences can be permuted in up to 1006 different ways. This large number of permutations cannot be represented in a single library of DNA sequences. Accordingly, it is contemplated that multiple cycles of DNA shuffling and selection may be required depending on the length of the sequence and the sequence diversity desired.

Error-prone PCR, in contrast, keeps all the selected CDRs in the same relative sequence, generating a much smaller mutant cloud.

The template polynucleotide which may be used in the methods of this invention may be DNA or RNA. It may be of various lengths depending on the size of the gene or shorter or smaller polynucleotide to be recombined or reassembled. Preferably, the template polynucleotide is from 50 bp to 50 kb. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest can be used in the methods of this invention, and in fact have been successfully used.

The template polynucleotide may be obtained by amplification using the PCR reaction (USPN 4,683,202 and USPN 4,683,195) or other amplification or cloning methods. However, the removal of free primers from the PCR products before subjecting them to pooling of the PCR products and sexual PCR may provide more efficient results. Failure to
5 adequately remove the primers from the original pool before sexual PCR can lead to a low frequency of crossover clones.

The template polynucleotide often should be double-stranded. A double-stranded nucleic acid molecule is recommended to ensure that regions of the resulting single-stranded polynucleotides are complementary to each other and thus can hybridize to form a
10 double-stranded molecule.

It is contemplated that single-stranded or double-stranded nucleic acid polynucleotides having regions of identity to the template polynucleotide and regions of heterology to the template polynucleotide may be added to the template polynucleotide, at this step. It is also contemplated that two different but related polynucleotide templates can
15 be mixed at this step.

The double-stranded polynucleotide template and any added double-or single-stranded polynucleotides are subjected to sexual PCR which includes slowing or halting to provide a mixture of from about 5 bp to 5 kb or more. Preferably the size of the random polynucleotides is from about 10 bp to 1000 bp, more preferably the size of the
20 polynucleotides is from about 20 bp to 500 bp.

Alternatively, it is also contemplated that double-stranded nucleic acid having multiple nicks may be used in the methods of this invention. A nick is a break in one strand of the double-stranded nucleic acid. The distance between such nicks is preferably 5 bp to 5 kb, more preferably between 10 bp to 1000 bp. This can provide areas of self-priming to
25 produce shorter or smaller polynucleotides to be included with the polynucleotides resulting from random primers, for example.

The concentration of any one specific polynucleotide will not be greater than 1% by weight of the total polynucleotides, more preferably the concentration of any one specific nucleic acid sequence will not be greater than 0.1% by weight of the total nucleic acid.

The number of different specific polynucleotides in the mixture will be at least about 100, preferably at least about 500, and more preferably at least about 1000.
30

At this step single-stranded or double-stranded polynucleotides, either synthetic or natural, may be added to the random double-stranded shorter or smaller polynucleotides in order to increase the heterogeneity of the mixture of polynucleotides.

5 It is also contemplated that populations of double-stranded randomly broken polynucleotides may be mixed or combined at this step with the polynucleotides from the sexual PCR process and optionally subjected to one or more additional sexual PCR cycles.

10 Where insertion of mutations into the template polynucleotide is desired, single-stranded or double-stranded polynucleotides having a region of identity to the template polynucleotide and a region of heterology to the template polynucleotide may be added in a 20 fold excess by weight as compared to the total nucleic acid, more preferably the single-stranded polynucleotides may be added in a 10 fold excess by weight as compared to the total nucleic acid.

15 Where a mixture of different but related template polynucleotides is desired, populations of polynucleotides from each of the templates may be combined at a ratio of less than about 1:100, more preferably the ratio is less than about 1:40. For example, a backcross of the wild-type polynucleotide with a population of mutated polynucleotide may be desired to eliminate neutral mutations (e.g., mutations yielding an insubstantial alteration in the phenotypic property being selected for). In such an example, the ratio of randomly provided wild-type polynucleotides which may be added to the randomly provided sexual PCR cycle 20 hybrid polynucleotides is approximately 1:1 to about 100:1, and more preferably from 1:1 to 40:1.

25 The mixed population of random polynucleotides are denatured to form single-stranded polynucleotides and then re-annealed. Only those single-stranded polynucleotides having regions of homology with other single-stranded polynucleotides will re-anneal.

30 The random polynucleotides may be denatured by heating. One skilled in the art could determine the conditions necessary to completely denature the double-stranded nucleic acid. Preferably the temperature is from 80 °C to 100 °C, more preferably the temperature is from 90 °C to 96 °C. other methods which may be used to denature the polynucleotides include pressure (36) and pH.

The polynucleotides may be re-annealed by cooling. Preferably the temperature is from 20 °C to 75 °C, more preferably the temperature is from 40 °C to 65 °C. If a high frequency of crossovers is needed based on an average of only 4 consecutive bases of homology, recombination can be forced by using a low annealing temperature, although the process becomes more difficult. The degree of renaturation which occurs will depend on the degree of homology between the population of single-stranded polynucleotides.

Renaturation can be accelerated by the addition of polyethylene glycol ("PEG") or salt. The salt concentration is preferably from 0 mM to 200 mM, more preferably the salt concentration is from 10 mM to 100 mM. The salt may be KCl or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%.

The annealed polynucleotides are next incubated in the presence of a nucleic acid polymerase and dNTP's (i.e. dATP, dCTP, dGTP and dTTP). The nucleic acid polymerase may be the Klenow fragment, the Taq polymerase or any other DNA polymerase known in the art.

The approach to be used for the assembly depends on the minimum degree of homology that should still yield crossovers. If the areas of identity are large, Taq polymerase can be used with an annealing temperature of between 45-65 °C. If the areas of identity are small, Klenow polymerase can be used with an annealing temperature of between 20-30 °C. One skilled in the art could vary the temperature of annealing to increase the number of cross-overs achieved.

The polymerase may be added to the random polynucleotides prior to annealing, simultaneously with annealing or after annealing.

The cycle of denaturation, renaturation and incubation in the presence of polymerase is referred to herein as shuffling or reassembly of the nucleic acid. This cycle is repeated for a desired number of times. Preferably the cycle is repeated from 2 to 50 times, more preferably the sequence is repeated from 10 to 40 times.

The resulting nucleic acid is a larger double-stranded polynucleotide of from about 50 bp to about 100 kb, preferably the larger polynucleotide is from 500 bp to 50 kb.

This larger polynucleotides may contain a number of copies of a polynucleotide having the same size as the template polynucleotide in tandem. This concatemeric polynucleotide is then denatured into single copies of the template polynucleotide. The result

will be a population of polynucleotides of approximately the same size as the template polynucleotide. The population will be a mixed population where single or double-stranded polynucleotides having an area of identity and an area of heterology have been added to the template polynucleotide prior to shuffling. These polynucleotides are then cloned into the appropriate vector and the ligation mixture used to transform bacteria.

It is contemplated that the single polynucleotides may be obtained from the larger concatemeric polynucleotide by amplification of the single polynucleotide prior to cloning by a variety of methods including PCR (USPN 4,683,195 and USPN 4,683,202), rather than by digestion of the concatemer.

The vector used for cloning is not critical provided that it will accept a polynucleotide of the desired size. If expression of the particular polynucleotide is desired, the cloning vehicle should further comprise transcription and translation signals next to the site of insertion of the polynucleotide to allow expression of the polynucleotide in the host cell. Preferred vectors include the pUC series and the pBR series of plasmids.

The resulting bacterial population will include a number of recombinant polynucleotides having random mutations. This mixed population may be tested to identify the desired recombinant polynucleotides. The method of selection will depend on the polynucleotide desired.

For example, if a polynucleotide which encodes a protein with increased binding efficiency to a ligand is desired, the proteins expressed by each of the portions of the polynucleotides in the population or library may be tested for their ability to bind to the ligand by methods known in the art (i.e. panning, affinity chromatography). If a polynucleotide which encodes for a protein with increased drug resistance is desired, the proteins expressed by each of the polynucleotides in the population or library may be tested for their ability to confer drug resistance to the host organism. One skilled in the art, given knowledge of the desired protein, could readily test the population to identify polynucleotides which confer the desired properties onto the protein.

It is contemplated that one skilled in the art could use a phage display system in which fragments of the protein are expressed as fusion proteins on the phage surface (Pharmacia, Milwaukee WI). The recombinant DNA molecules are cloned into the phage DNA at a site which results in the transcription of a fusion protein a portion of which is encoded by the recombinant DNA molecule. The phage containing the recombinant nucleic

acid molecule undergoes replication and transcription in the cell. The leader sequence of the fusion protein directs the transport of the fusion protein to the tip of the phage particle. Thus the fusion protein which is partially encoded by the recombinant DNA molecule is displayed on the phage particle for detection and selection by the methods described above.

5 It is further contemplated that a number of cycles of nucleic acid shuffling may be conducted with polynucleotides from a sub-population of the first population, which sub-population contains DNA encoding the desired recombinant protein. In this manner, proteins with even higher binding affinities or enzymatic activity could be achieved.

10 It is also contemplated that a number of cycles of nucleic acid shuffling may be conducted with a mixture of wild-type polynucleotides and a sub-population of nucleic acid from the first or subsequent rounds of nucleic acid shuffling in order to remove any silent mutations from the sub-population.

15 Any source of nucleic acid, in purified form can be utilized as the starting nucleic acid. Thus the process may employ DNA or RNA including messenger RNA, which DNA or RNA may be single or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. The nucleic acid sequence may be of various lengths depending on the size of the nucleic acid sequence to be mutated. Preferably the specific nucleic acid sequence is from 50 to 50000 base pairs. It is contemplated that entire vectors containing the nucleic acid encoding the protein of interest may be used in the methods of
20 this invention.

The nucleic acid may be obtained from any source, for example, from plasmids such a pBR322, from cloned DNA or RNA or from natural DNA or RNA from any source including bacteria, yeast, viruses and higher organisms such as plants or animals. DNA or RNA may be extracted from blood or tissue material. The template polynucleotide may be
25 obtained by amplification using the polynucleotide chain reaction (PCR, see USPN 4,683,202 and USPN 4,683,195). Alternatively, the polynucleotide may be present in a vector present in a cell and sufficient nucleic acid may be obtained by culturing the cell and extracting the nucleic acid from the cell by methods known in the art.

30 Any specific nucleic acid sequence can be used to produce the population of hybrids by the present process. It is only necessary that a small population of hybrid sequences of the specific nucleic acid sequence exist or be created prior to the present process.

The initial small population of the specific nucleic acid sequences having mutations may be created by a number of different methods. Mutations may be created by error-prone PCR. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. Alternatively, mutations can be introduced into the template polynucleotide by oligonucleotide-directed mutagenesis. In oligonucleotide-directed mutagenesis, a short sequence of the polynucleotide is removed from the polynucleotide using restriction enzyme digestion and is replaced with a synthetic polynucleotide in which various bases have been altered from the original sequence. The polynucleotide sequence can also be altered by chemical mutagenesis. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. Other agents which are analogues of nucleotide precursors include nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Generally, these agents are added to the PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used. Random mutagenesis of the polynucleotide sequence can also be achieved by irradiation with X-rays or ultraviolet light. Generally, plasmid polynucleotides so mutagenized are introduced into *E. coli* and propagated as a pool or library of hybrid plasmids.

Alternatively the small mixed population of specific nucleic acids may be found in nature in that they may consist of different alleles of the same gene or the same gene from different related species (i.e., cognate genes). Alternatively, they may be related DNA sequences found within one species, for example, the immunoglobulin genes.

Once the mixed population of the specific nucleic acid sequences is generated, the polynucleotides can be used directly or inserted into an appropriate cloning vector, using techniques well-known in the art.

The choice of vector depends on the size of the polynucleotide sequence and the host cell to be employed in the methods of this invention. The templates of this invention may be plasmids, phages, cosmids, phagemids, viruses (e.g., retroviruses, parainfluenzavirus, herpesviruses, reoviruses, paramyxoviruses, and the like), or selected portions thereof (e.g., coat protein, spike glycoprotein, capsid protein). For example, cosmids and phagemids are preferred where the specific nucleic acid sequence to be mutated is larger because these vectors are able to stably propagate large polynucleotides.

If the mixed population of the specific nucleic acid sequence is cloned into a vector it can be clonally amplified by inserting each vector into a host cell and allowing the host cell to amplify the vector. This is referred to as clonal amplification because while the absolute number of nucleic acid sequences increases, the number of hybrids does not increase. Utility can be readily determined by screening expressed polypeptides.

The DNA shuffling method of this invention can be performed blindly on a pool of unknown sequences. By adding to the reassembly mixture oligonucleotides (with ends that are homologous to the sequences being reassembled) any sequence mixture can be incorporated at any specific position into another sequence mixture. Thus, it is contemplated that mixtures of synthetic oligonucleotides, PCR polynucleotides or even whole genes can be mixed into another sequence library at defined positions. The insertion of one sequence (mixture) is independent from the insertion of a sequence in another part of the template. Thus, the degree of recombination, the homology required, and the diversity of the library can be independently and simultaneously varied along the length of the reassembled DNA.

This approach of mixing two genes may be useful for the humanization of antibodies from murine hybridomas. The approach of mixing two genes or inserting alternative sequences into genes may be useful for any therapeutically used protein, for example, interleukin I, antibodies, tPA and growth hormone. The approach may also be useful in any nucleic acid for example, promoters or introns or 3' untranslated region or 5' untranslated regions of genes to increase expression or alter specificity of expression of proteins. The approach may also be used to mutate ribozymes or aptamers.

Shuffling requires the presence of homologous regions separating regions of diversity. Scaffold-like protein structures may be particularly suitable for shuffling. The conserved scaffold determines the overall folding by self-association, while displaying relatively unrestricted loops that mediate the specific binding. Examples of such scaffolds are the immunoglobulin beta-barrel, and the four-helix bundle which are well-known in the art. This shuffling can be used to create scaffold-like proteins with various combinations of mutated sequences for binding.

In vitro Shuffling

The equivalents of some standard genetic matings may also be performed by shuffling *in vitro*. For example, a "molecular backcross" can be performed by repeatedly mixing the hybrid's nucleic acid with the wild-type nucleic acid while selecting for the

mutations of interest. As in traditional breeding, this approach can be used to combine phenotypes from different sources into a background of choice. It is useful, for example, for the removal of neutral mutations that affect unselected characteristics (i.e. immunogenicity). Thus it can be useful to determine which mutations in a protein are involved in the enhanced biological activity and which are not, an advantage which cannot be achieved by error-prone mutagenesis or cassette mutagenesis methods.

Large, functional genes can be assembled correctly from a mixture of small random polynucleotides. This reaction may be of use for the reassembly of genes from the highly fragmented DNA of fossils. In addition random nucleic acid fragments from fossils may be combined with polynucleotides from similar genes from related species.

It is also contemplated that the method of this invention can be used for the *in vitro* amplification of a whole genome from a single cell as is needed for a variety of research and diagnostic applications. DNA amplification by PCR is in practice limited to a length of about 40 kb. Amplification of a whole genome such as that of *E. coli* (5, 000 kb) by PCR would require about 250 primers yielding 125 forty kb polynucleotides. This approach is not practical due to the unavailability of sufficient sequence data. On the other hand, random production of polynucleotides of the genome with sexual PCR cycles, followed by gel purification of small polynucleotides will provide a multitude of possible primers. Use of this mix of random small polynucleotides as primers in a PCR reaction alone or with the whole genome as the template should result in an inverse chain reaction with the theoretical endpoint of a single concatamer containing many copies of the genome.

100 fold amplification in the copy number and an average polynucleotide size of greater than 50 kb may be obtained when only random polynucleotides are used. It is thought that the larger concatamer is generated by overlap of many smaller polynucleotides. The quality of specific PCR products obtained using synthetic primers will be indistinguishable from the product obtained from unamplified DNA. It is expected that this approach will be useful for the mapping of genomes.

The polynucleotide to be shuffled can be produced as random or non-random polynucleotides, at the discretion of the practitioner. Moreover, this invention provides a method of shuffling that is applicable to a wide range of polynucleotide sizes and types, including the step of generating polynucleotide monomers to be used as building blocks in the reassembly of a larger polynucleotide. For example, the building blocks can be

fragments of genes or they can be comprised of entire genes or gene pathways, or any combination thereof.

In Vivo Shuffling

5 In an embodiment of *in vivo* shuffling, the mixed population of the specific nucleic acid sequence is introduced into bacterial or eukaryotic cells under conditions such that at least two different nucleic acid sequences are present in each host cell. The polynucleotides can be introduced into the host cells by a variety of different methods. The host cells can be transformed with the smaller polynucleotides using methods known in the art, for example treatment with calcium chloride. If the polynucleotides are inserted into a phage genome, the
10 host cell can be transfected with the recombinant phage genome having the specific nucleic acid sequences. Alternatively, the nucleic acid sequences can be introduced into the host cell using electroporation, transfection, lipofection, biolistics, conjugation, and the like.

In general, in this embodiment, the specific nucleic acids sequences will be present in vectors which are capable of stably replicating the sequence in the host cell. In addition, it is
15 contemplated that the vectors will encode a marker gene such that host cells having the vector can be selected. This ensures that the mutated specific nucleic acid sequence can be recovered after introduction into the host cell. However, it is contemplated that the entire mixed population of the specific nucleic acid sequences need not be present on a vector sequence. Rather only a sufficient number of sequences need be cloned into vectors to
20 ensure that after introduction of the polynucleotides into the host cells each host cell contains one vector having at least one specific nucleic acid sequence present therein. It is also contemplated that rather than having a subset of the population of the specific nucleic acids sequences cloned into vectors, this subset may be already stably integrated into the host cell.

It has been found that when two polynucleotides which have regions of identity are
25 inserted into the host cells homologous recombination occurs between the two polynucleotides. Such recombination between the two mutated specific nucleic acid sequences will result in the production of double or triple hybrids in some situations.

It has also been found that the frequency of recombination is increased if some of the mutated specific nucleic acid sequences are present on linear nucleic acid molecules. In one
30 aspect, some of the specific nucleic acid sequences are present on linear polynucleotides.

After transformation, the host cell transformants are placed under selection to identify those host cell transformants which contain mutated specific nucleic acid sequences having

the qualities desired. For example, if increased resistance to a particular drug is desired then the transformed host cells may be subjected to increased concentrations of the particular drug and those transformants producing mutated proteins able to confer increased drug resistance will be selected. If the enhanced ability of a particular protein to bind to a receptor is desired, then expression of the protein can be induced from the transformants and the resulting protein assayed in a ligand binding assay by methods known in the art to identify that subset of the mutated population which shows enhanced binding to the ligand. Alternatively, the protein can be expressed in another system to ensure proper processing.

Once a subset of the first recombined specific nucleic acid sequences (daughter sequences) having the desired characteristics are identified, they are then subject to a second round of recombination.

In the second cycle of recombination, the recombined specific nucleic acid sequences may be mixed with the original mutated specific nucleic acid sequences (parent sequences) and the cycle repeated as described above. In this way a set of second recombined specific nucleic acids sequences can be identified which have enhanced characteristics or encode for proteins having enhanced properties. This cycle can be repeated a number of times as desired.

It is also contemplated that in the second or subsequent recombination cycle, a backcross can be performed. A molecular backcross can be performed by mixing the desired specific nucleic acid sequences with a large number of the wild-type sequence, such that at least one wild-type nucleic acid sequence and a mutated nucleic acid sequence are present in the same host cell after transformation. Recombination with the wild-type specific nucleic acid sequence will eliminate those neutral mutations that may affect unselected characteristics such as immunogenicity but not the selected characteristics.

In another embodiment of this invention, it is contemplated that during the first round a subset of the specific nucleic acid sequences can be generated as smaller polynucleotides by slowing or halting their PCR amplification prior to introduction into the host cell. The size of the polynucleotides must be large enough to contain some regions of identity with the other sequences so as to homologously recombine with the other sequences. The size of the polynucleotides will range from 0.03 kb to 100 kb more preferably from 0.2 kb to 10 kb. It is also contemplated that in subsequent rounds, all of the specific nucleic acid sequences

other than the sequences selected from the previous round may be utilized to generate PCR polynucleotides prior to introduction into the host cells.

The shorter polynucleotide sequences can be single-stranded or double-stranded. If the sequences were originally single-stranded and have become double-stranded they can be denatured with heat, chemicals or enzymes prior to insertion into the host cell. The reaction conditions suitable for separating the strands of nucleic acid are well known in the art.

The steps of this process can be repeated indefinitely, being limited only by the number of possible hybrids which can be achieved. After a certain number of cycles, all possible hybrids will have been achieved and further cycles are redundant.

In an embodiment the same mutated template nucleic acid is repeatedly recombined and the resulting recombinants selected for the desired characteristic.

Therefore, the initial pool or population of mutated template nucleic acid is cloned into a vector capable of replicating in a bacteria such as *E. coli*. The particular vector is not essential, so long as it is capable of autonomous replication in *E. coli*. In a preferred embodiment, the vector is designed to allow the expression and production of any protein encoded by the mutated specific nucleic acid linked to the vector. It is also preferred that the vector contain a gene encoding for a selectable marker.

The population of vectors containing the pool of mutated nucleic acid sequences is introduced into the *E. coli* host cells. The vector nucleic acid sequences may be introduced by transformation, transfection or infection in the case of phage. The concentration of vectors used to transform the bacteria is such that a number of vectors is introduced into each cell. Once present in the cell, the efficiency of homologous recombination is such that homologous recombination occurs between the various vectors. This results in the generation of hybrids (daughters) having a combination of mutations which differ from the original parent mutated sequences.

The host cells are then clonally replicated and selected for the marker gene present on the vector. Only those cells having a plasmid will grow under the selection.

The host cells which contain a vector are then tested for the presence of favorable mutations. Such testing may consist of placing the cells under selective pressure, for example, if the gene to be selected is an improved drug resistance gene. If the vector allows expression of the protein encoded by the mutated nucleic acid sequence, then such selection may include allowing expression of the protein so encoded, isolation of the protein and

testing of the protein to determine whether, for example, it binds with increased efficiency to the ligand of interest.

Once a particular daughter mutated nucleic acid sequence has been identified which confers the desired characteristics, the nucleic acid is isolated either already linked to the vector or separated from the vector. This nucleic acid is then mixed with the first or parent
5 population of nucleic acids and the cycle is repeated.

It has been shown that by this method nucleic acid sequences having enhanced desired properties can be selected.

In an alternate embodiment, the first generation of hybrids are retained in the cells and the parental mutated sequences are added again to the cells. Accordingly, the first cycle
10 of Embodiment I is conducted as described above. However, after the daughter nucleic acid sequences are identified, the host cells containing these sequences are retained.

The parent mutated specific nucleic acid population, either as polynucleotides or cloned into the same vector is introduced into the host cells already containing the daughter
15 nucleic acids. Recombination is allowed to occur in the cells and the next generation of recombinants, or granddaughters are selected by the methods described above.

This cycle can be repeated a number of times until the nucleic acid or peptide having the desired characteristics is obtained. It is contemplated that in subsequent cycles, the population of mutated sequences which are added to the preferred hybrids may come from
20 the parental hybrids or any subsequent generation.

In an alternative embodiment, the invention provides a method of conducting a "molecular" backcross of the obtained recombinant specific nucleic acid in order to eliminate any neutral mutations. Neutral mutations are those mutations which do not confer onto the nucleic acid or peptide the desired properties. Such mutations may however confer on the
25 nucleic acid or peptide undesirable characteristics. Accordingly, it is desirable to eliminate such neutral mutations. The method of this invention provide a means of doing so.

In this embodiment, after the hybrid nucleic acid, having the desired characteristics, is obtained by the methods of the embodiments, the nucleic acid, the vector having the nucleic acid or the host cell containing the vector and nucleic acid is isolated.

The nucleic acid or vector is then introduced into the host cell with a large excess of the wild-type nucleic acid. The nucleic acid of the hybrid and the nucleic acid of the wild-type sequence are allowed to recombine. The resulting recombinants are placed under
30

the same selection as the hybrid nucleic acid. Only those recombinants which retained the desired characteristics will be selected. Any silent mutations which do not provide the desired characteristics will be lost through recombination with the wild-type DNA. This cycle can be repeated a number of times until all of the silent mutations are eliminated.

5 Thus the methods of this invention can be used in a molecular backcross to eliminate unnecessary or silent mutations.

Exonuclease-Mediated Reassembly

10 In a particular embodiment, this invention provides for a method for shuffling, assembling, reassembling, recombining, &/or concatenating at least two polynucleotides to form a progeny polynucleotide (e.g. a chimeric progeny polynucleotide that can be expressed to produce a polypeptide or a gene pathway). In a particular embodiment, a double stranded polynucleotide end (e.g. two single stranded sequences hybridized to each other as hybridization partners) is treated with an exonuclease to liberate nucleotides from one of the two strands, leaving the remaining strand free of its original partner so that, if desired, the
15 remaining strand may be used to achieve hybridization to another partner.

 In a particular aspect, a double stranded polynucleotide end (that may be part of - or connected to - a polynucleotide or a nonpolynucleotide sequence) is subjected to a source of exonuclease activity. Serviceable sources of exonuclease activity may be an enzyme with 3' exonuclease activity, an enzyme with 5' exonuclease activity, an enzyme with both 3' exonuclease activity and 5' exonuclease activity, and any combination thereof. An
20 exonuclease can be used to liberate nucleotides from one or both ends of a linear double stranded polynucleotide, and from one to all ends of a branched polynucleotide having more than two ends. The mechanism of action of this liberation is believed to be comprised of an enzymatically-catalyzed hydrolysis of terminal nucleotides, and can be allowed to proceed in
25 a time-dependent fashion, allowing experimental control of the progression of the enzymatic process.

 By contrast, a non-enzymatic step may be used to shuffle, assemble, reassemble, recombine, and/or concatenate polynucleotide building blocks that is comprised of subjecting a working sample to denaturing (or "melting") conditions (for example, by changing
30 temperature, pH, and /or salinity conditions) so as to melt a working set of double stranded polynucleotides into single polynucleotide strands. For shuffling, it is desirable that the single polynucleotide strands participate to some extent in annealment with different

hybridization partners (i.e. and not merely revert to exclusive reannealment between what were former partners before the denaturation step). The presence of the former hybridization partners in the reaction vessel, however, does not preclude, and may sometimes even favor, reannealment of a single stranded polynucleotide with its former partner, to recreate an original double stranded polynucleotide.

In contrast to this non-enzymatic shuffling step comprised of subjecting double stranded polynucleotide building blocks to denaturation, followed by annealment, the instant invention further provides an exonuclease-based approach requiring no denaturation – rather, the avoidance of denaturing conditions and the maintenance of double stranded polynucleotide substrates in annealed (i.e. non-denatured) state are necessary conditions for the action of exonucleases (e.g., exonuclease III and red alpha gene product). Additionally in contrast, the generation of single stranded polynucleotide sequences capable of hybridizing to other single stranded polynucleotide sequences is the result of covalent cleavage – and hence sequence destruction - in one of the hybridization partners. For example, an exonuclease III enzyme may be used to enzymatically liberate 3' terminal nucleotides in one hybridization strand (to achieve covalent hydrolysis in that polynucleotide strand); and this favors hybridization of the remaining single strand to a new partner (since its former partner was subjected to covalent cleavage).

By way of further illustration, a specific exonuclease, namely exonuclease III is provided herein as an example of a 3' exonuclease; however, other exonucleases may also be used, including enzymes with 5' exonuclease activity and enzymes with 3' exonuclease activity, and including enzymes not yet discovered and enzymes not yet developed. It is particularly appreciated that enzymes can be discovered, optimized (e.g. engineered by directed evolution), or both discovered and optimized specifically for the instantly disclosed approach that have more optimal rates &/or more highly specific activities &/or greater lack of unwanted activities. In fact it is expected that the instant invention may encourage the discovery &/or development of such designer enzymes. In sum, this invention may be practiced with a variety of currently available exonuclease enzymes, as well enzymes not yet discovered and enzymes not yet developed.

The exonuclease action of exonuclease III requires a working double stranded polynucleotide end that is either blunt or has a 5' overhang, and the exonuclease action is comprised of enzymatically liberating 3' terminal nucleotides, leaving a single stranded 5'

end that becomes longer and longer as the exonuclease action proceeds (see Figure 1). Any 5' overhangs produced by this approach may be used to hybridize to another single stranded polynucleotide sequence (which may also be a single stranded polynucleotide or a terminal overhang of a partially double stranded polynucleotide) that shares enough homology to allow hybridization. The ability of these exonuclease III-generated single stranded sequences (e.g. in 5' overhangs) to hybridize to other single stranded sequences allows two or more polynucleotides to be shuffled, assembled, reassembled, &/or concatenated.

Furthermore, it is appreciated that one can protect the end of a double stranded polynucleotide or render it susceptible to a desired enzymatic action of a serviceable exonuclease as necessary. For example, a double stranded polynucleotide end having a 3' overhang is not susceptible to the exonuclease action of exonuclease III. However, it may be rendered susceptible to the exonuclease action of exonuclease III by a variety of means; for example, it may be blunted by treatment with a polymerase, cleaved to provide a blunt end or a 5' overhang, joined (ligated or hybridized) to another double stranded polynucleotide to provide a blunt end or a 5' overhang, hybridized to a single stranded polynucleotide to provide a blunt end or a 5' overhang, or modified by any of a variety of means).

According to one aspect, an exonuclease may be allowed to act on one or on both ends of a linear double stranded polynucleotide and proceed to completion, to near completion, or to partial completion. When the exonuclease action is allowed to go to completion, the result will be that the length of each 5' overhang will be extend far towards the middle region of the polynucleotide in the direction of what might be considered a "rendezvous point" (which may be somewhere near the polynucleotide midpoint). Ultimately, this results in the production of single stranded polynucleotides (that can become dissociated) that are each about half the length of the original double stranded polynucleotide (see Figure 1). Alternatively, an exonuclease-mediated reaction can be terminated before proceeding to completion.

Thus this exonuclease-mediated approach is serviceable for shuffling, assembling &/or reassembling, recombining, and concatenating polynucleotide building blocks, which polynucleotide building blocks can be up to ten bases long or tens of bases long or hundreds of bases long or thousands of bases long or tens of thousands of bases long or hundreds of thousands of bases long or millions of bases long or even longer.

This exonuclease-mediated approach is based on the action of double stranded DNA specific exodeoxyribonuclease activity of *E. coli* exonuclease III. Substrates for exonuclease III may be generated by subjecting a double stranded polynucleotide to fragmentation. Fragmentation may be achieved by mechanical means (e.g., shearing, sonication, etc.), by enzymatic means (e.g. using restriction enzymes), and by any combination thereof. Fragments of a larger polynucleotide may also be generated by polymerase-mediated synthesis.

Exonuclease III is a 28K monomeric enzyme, product of the *xthA* gene of *E. coli* with four known activities: exodeoxyribonuclease (alternatively referred to as exonuclease herein), RNaseH, DNA-3'-phosphatase, and AP endonuclease. The exodeoxyribonuclease activity is specific for double stranded DNA. The mechanism of action is thought to involve enzymatic hydrolysis of DNA from a 3' end progressively towards a 5' direction, with formation of nucleoside 5'-phosphates and a residual single strand. The enzyme does not display efficient hydrolysis of single stranded DNA, single-stranded RNA, or double-stranded RNA; however it degrades RNA in an DNA-RNA hybrid releasing nucleoside 5'-phosphates. The enzyme also releases inorganic phosphate specifically from 3'phosphomonoester groups on DNA, but not from RNA or short oligonucleotides. Removal of these groups converts the terminus into a primer for DNA polymerase action.

Additional examples of enzymes with exonuclease activity include red-alpha and venom phosphodiesterases. Red alpha (*red α*) gene product (also referred to as lambda exonuclease) is of bacteriophage λ origin. The *red α* gene is transcribed from the leftward promoter and its product is involved (24 kD) in recombination. Red alpha gene product acts processively from 5'-phosphorylated termini to liberate mononucleotides from duplex DNA (Takahashi & Kobayashi, 1990). Venom phosphodiesterases (Laskowski, 1980) is capable of rapidly opening supercoiled DNA.

Non-Stochastic Ligation Reassembly

In one aspect, the present invention provides a non-stochastic method termed synthetic ligation reassembly (SLR), that is somewhat related to stochastic shuffling, save that the nucleic acid building blocks are not shuffled or concatenated or chimerized randomly, but rather are assembled non-stochastically.

A particularly glaring difference is that the instant SLR method does not depend on the presence of a high level of homology between polynucleotides to be shuffled. In contrast, prior methods, particularly prior stochastic shuffling methods require that presence of a high level of homology, particularly at coupling sites, between polynucleotides to be shuffled. Accordingly these prior methods favor the regeneration of the original progenitor molecules, and are suboptimal for generating large numbers of novel progeny chimeras, particularly full-length progenies. The instant invention, on the other hand, can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over 10^{100} different chimeras. Conceivably, SLR can even be used to generate libraries comprised of over 10^{1000} different progeny chimeras with (no upper limit in sight).

Thus, in one aspect, the present invention provides a method, which method is non-stochastic, of producing a set of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design, which method is comprised of the steps of generating by design a plurality of specific nucleic acid building blocks having serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.

The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be "serviceable" for this type of ordered assembly if they enable the building blocks to be coupled in predetermined orders. Thus, in one aspect, the overall assembly order in which the nucleic acid building blocks can be coupled is specified by the design of the ligatable ends and, if more than one assembly step is to be used, then the overall assembly order in which the nucleic acid building blocks can be coupled is also specified by the sequential order of the assembly step(s). Figure 4, Panel C illustrates an exemplary assembly process comprised of 2 sequential steps to achieve a designed (non-stochastic) overall assembly order for five nucleic acid building blocks. In a preferred embodiment of this invention, the annealed building pieces are treated with an enzyme, such as a ligase (e.g. T4 DNA ligase), achieve covalent bonding of the building pieces.

In one aspect, the design of nucleic acid building blocks is obtained upon analysis of the sequences of a set of progenitor nucleic acid templates that serve as a basis for producing a progeny set of finalized chimeric nucleic acid molecules. These progenitor nucleic acid templates thus serve as a source of sequence information that aids in the design of the nucleic acid building blocks that are to be mutagenized, i.e. chimerized or shuffled.

In one exemplification, this invention provides for the chimerization of a family of related genes and their encoded family of related products. In a particular exemplification, the encoded products are enzymes. As a representative list of families of enzymes which may be mutagenized in accordance with the aspects of the present invention, there may be mentioned, the following enzymes and their functions:

1 Lipase/Esterase

- a. Enantioselective hydrolysis of esters (lipids)/ thioesters
 - 1) Resolution of racemic mixtures
 - 2) Synthesis of optically active acids or alcohols from *meso*-diesters
- b. Selective syntheses
 - 1) Regiospecific hydrolysis of carbohydrate esters
 - 2) Selective hydrolysis of cyclic secondary alcohols
- c. Synthesis of optically active esters, lactones, acids, alcohols
 - 1) Transesterification of activated/nonactivated esters
 - 2) Interesterification
 - 3) Optically active lactones from hydroxyesters
 - 4) Regio- and enantioselective ring opening of anhydrides
- d. Detergents
- e. Fat/Oil conversion
- f. Cheese ripening

2 Protease

- a. Ester/amide synthesis
- b. Peptide synthesis
- c. Resolution of racemic mixtures of amino acid esters
- d. Synthesis of non-natural amino acids
- e. Detergents/protein hydrolysis

3 Glycosidase/Glycosyl transferase

- a. Sugar/polymer synthesis
- b. Cleavage of glycosidic linkages to form mono, di-and oligosaccharides
- c. Synthesis of complex oligosaccharides
- d. Glycoside synthesis using UDP-galactosyl transferase
- e. Transglycosylation of disaccharides, glycosyl fluorides, aryl galactosides
- f. Glycosyl transfer in oligosaccharide synthesis
- g. Diastereoselective cleavage of β -glucosylsulfoxides
- h. Asymmetric glycosylations
- i. Food processing
- j. Paper processing

4 Phosphatase/Kinase

- a. Synthesis/hydrolysis of phosphate esters
 - 1) Regio-, enantioselective phosphorylation
 - 2) Introduction of phosphate esters
 - 3) Synthesize phospholipid precursors
 - 4) Controlled polynucleotide synthesis